BRAIN H^+/CO_2 SENSING AND CONTROL BY GLIAL CELLS

Alexander V Gourine[1] and Nicholas Dale[2]

[1]*Centre for Cardiovascular and Metabolic Neuroscience, Department of Neuroscience, Physiology and Pharmacology, University College London, London WC1E 6BT, UK*

[2]*School of Life Sciences, University of Warwick, Coventry, CV4 7AL, UK*

Abstract

Maintenance of constant brain pH is critically important for uninterrupted activity of individual neurons, effective communication within the neuronal circuits, and, thus, efficient processing of information by the brain. This review article focuses on how glial cells detect and respond to changes in brain tissue pH and concentration of CO_2, and then trigger systemic and local adaptive mechanisms that ensure a stable milieu for the operation of brain circuits. We give a detailed account of the cellular and molecular mechanisms underlying sensitivity of glial cells to H^+ and CO_2 and discuss the role of glial chemosensitivity and signalling in operation of three key mechanisms that work in concert to keep the brain pH constant. We discuss evidence suggesting that astrocytes and marginal glial cells of the brainstem are critically important for central respiratory CO_2 chemoreception, – a fundamental physiological mechanism that regulates breathing in accord with changes in blood and brain pH and partial pressure of CO_2 in order to maintain systemic pH homeostasis. We review evidence suggesting that astrocytes are also responsible for the maintenance of local brain tissue extracellular pH, in conditions of variable acid loads associated with changes in the neuronal activity, and discuss potential role of these glial cells in mediating the effects of CO_2 on cerebral vasculature.

Introduction

The high metabolic rate of the brain, associated with the activities of billions of nerve cells processing information, requires constant and optimal nutrient and oxygen supply, as well as effective elimination of metabolic waste. These processes are ensured by brain-specific mechanisms that control cerebral blood flow, metabolic substrate processing and delivery, maintenance of acid-base balance, and by systemic mechanisms controlling the circulation and breathing. There is growing evidence that reduced capacity of these mechanisms results in metabolic deficit, precipitates neuronal damage, contributes to cognitive decline and the development of dementia and neurodegenerative disease (Iadecola, 2004; Zlokovic, 2011; Iturria-Medina *et al.*, 2016; Nortley *et al.*, 2019; Iadecola & Gottesman, 2019; Lyros *et al.*, 2020; Hirunpattarasilp *et al.*, 2019; Kummer *et al.*, 2019).

Maintaining brain pH homeostasis is especially important to ensure uninterrupted activity of individual neurons and effective communication within neuronal circuits which makes the brain computation possible. The human brain generates 3.3 moles or ~75 litres of CO_2 per day, which corresponds to ~20% of total body CO_2 production. CO_2 and H_2O are in a dynamic equilibrium with H^+ and HCO_3^- and the rate of CO_2/acid production corresponds to the levels of neuronal activity and energy usage. All membrane, molecular and biochemical processes involved in synaptic transmission are sensitive to changes in pH, therefore, uncontrolled fluctuations in brain tissue CO_2/pH are detrimental to neuronal activity and function (Chesler, 2003). Disturbances of brain pH homeostasis have been implicated in the pathogenesis of several common neurological and psychiatric disorders such as epilepsy and schizophrenia (Hagihara et al., 2018). Brain aging is associated with progressive acidification and this process is facilitated by the development of Alzheimer's disease (Lyros *et al.*, 2020; Decker *et al.*, 2021). A strong correlation between full scale human intelligent quotient (IQ) and brain pH was reported in adolescents (7-13 years old boys), with lower pH associated with lower IQ scores (Rae et al., 1996).

This review article focuses on the mechanisms underlying sensitivity of glial cells to changes in brain tissue pH and CO_2 and physiological significance of this sensitivity. We discuss evidence suggesting that astrocytes and marginal glial cells of the brainstem play an important role in central respiratory CO_2 chemoreception, - the fundamental

brain mechanism that adjusts breathing in accord with changes in blood and brain pH and partial pressure of CO_2 (PCO_2) and, therefore, maintain systemic (arterial) pH homeostasis. We review recent evidence suggesting that astrocytes also contribute to the neuronal-activity dependent control of local brain tissue extracellular pH and discuss potential role of these glial cells in mediating the effects of CO_2 on cerebral vasculature.

Astrocytes in brief

Astrocytes are numerous brain glial cells that tile brain tissue and enwrap all penetrating and intracerebral (parenchymal) arterioles and capillaries. Astrocytes control the ionic environment of the neuropil and support synaptic activity by supplying neurons with a renewable source of transmitters (Khakh & Sofroniew, 2015). A single astrocyte may enwrap several neuronal somata (Halassa et al., 2007), make contacts with thousands of individual synapses (Bushong et al., 2002) and has a highly dynamic processes capable of altering the extracellular space (Henneberger et al., 2020). Astrocytes signal via mobilisation of intracellular Ca^{2+} and the release of gliotransmitters (such as ATP/adenosine, glutamate, D-serine) to modulate neuronal excitability, synaptic transmission, plasticity, and information processing (Araque et al., 2014). Astrocytes are extensively inter-connected into excitable networks via gap junctions and gliotransmitter release and, therefore, can potentially modulate the cellular activities across the neural networks (Fellin *et al.*, 2009; Clasadonte *et al.*, 2017).

There is emerging evidence that astrocytes function as versatile surveyors of the CNS metabolic milieu, equipped to detect metabolic threats, such as hypoxia (low oxygen), hypercapnia (high CO_2/acidification) and reduced brain perfusion (Angelova *et al.*, 2015; Gourine, 2005; Gourine *et al.*, 2005; Gourine *et al.*, 2010; Huckstepp *et al.*, 2010b; Kasymov *et al.*, 2013; Marina *et al.*, 2018; Marina *et al.*, 2020; Sheikhbahaei *et al.*, 2018b; Theparambil *et al.*, 2020; Turovsky *et al.*, 2016; Turovsky *et al.*, 2020; Wells *et al.*, 2015). Under conditions of increased metabolic demand, astrocytes that reside within the brain areas harbouring respiratory and cardiovascular control circuits can modulate the activities of these vital networks of neurons, and contribute to the development of the adaptive changes in breathing, heart rate and arterial blood pressure (Angelova *et al.*, 2015; Gourine *et al.*, 2005; Gourine *et al.*, 2010; Gourine & Funk, 2017; Marina *et al.*, 2018; Marina *et al.*, 2020; Sheikhbahaei *et al.*, 2018b).

CNS circuits controlling breathing and central respiratory CO_2 chemoreception

Cardiovascular and respiratory systems have evolved to serve the main function of delivering sufficient amounts of oxygen to, and removing CO_2 produced during metabolism from all tissues of the body. The neuronal circuits controlling breathing are located in the brainstem, - within bilaterally organized dorsal respiratory group and ventral respiratory column of neurons in the pons and the medulla oblongata (Smith *et al.*, 2013; Del Negro *et al.*, 2018). This extensive brainstem network is comprised of several functional divisions, including the pre-Bötzinger complex (preBötC) of neurons that generate the basic rhythm of breathing (Smith *et al.*, 2013; Del Negro *et al.*, 2018), and the group of neurons that populate the so-called retrotrapezoid nucleus (RTN), which in accord with the currently prevailing view primarily mediates the effects of CO_2 on breathing (Guyenet et al., 2019). Indeed, lesions placed within the RTN, silencing of the RTN neurons, or genetic deletion of pH sensitive receptors/channels expressed by the RTN neurons result in a substantial reduction of the respiratory response to CO_2 (Guyenet et al., 2019). However, significant reductions of CO_2 response could also be observed following lesions or inhibition of neurons in other areas of the brainstem, including the preBötC and the regions of the medullary raphe, suggesting that respiratory CO_2 chemosensitivity is distributed between several distinct groups of neurons, with several sites contributing to central CO_2 chemoreception, some located at a distance from the ventral medulla (Putnam *et al.*, 2004; Nattie & Li, 2012). It is also important to mention that peripheral arterial chemoreceptors in the carotid and aortic (in some species) bodies are responsible for about one-third of the ventilatory sensitivity to CO_2 and appear to play a significant role in controlling arterial pH/PCO_2 during resting breathing (Forster et al., 2008). Discussion of the mechanisms and the role played by the peripheral chemoreceptors in sensing arterial CO_2 and maintenance of systemic pH homeostasis is beyond the scope of this essay. Experimental evidence suggests that up to 70% of the ventilatory response to CO_2 remain in conditions when the inputs from peripheral chemoreceptors are blocked (see e.g. (Heeringa et al., 1979)), pointing to the critical importance of the brain mechanisms of respiratory CO_2 chemoreception.

From the early work of Hans Loeschcke and colleagues it was generally believed that in the brain CO_2 is detected via proxy of associated pH changes within the chemosensory regions of the brainstem (Loeschcke, 1982). However, there is evidence that the

brainstem acidification produced by CO_2 (respiratory acidosis) triggers much stronger respiratory responses compared to acidification induced at constant CO_2 (metabolic acidosis) (Shams, 1985), suggesting that the central respiratory chemoreception is mediated by distinct mechanisms that are sensitive to changes in pH and CO_2, and working in parallel.

Although respiratory activity is probably the most robust of all rhythmic behaviours, the mammalian central nervous circuitry that generates breathing is silent in the absence of CO_2 and requires a threshold level of CO_2 to be active. This suggests that the evolution of the respiratory CO_2 chemosensitivity in air breathing vertebrates was dictated primarily by the need of effective CO_2 elimination. The transition from water to air breathing made regulation of acid-base balance in body tissues more challenging, because the 7-fold greater concentration of O_2 in air as opposed to water led to evolution of lower ventilation rates in air breathing animals and, thus, lower rates of CO_2 excretion and higher levels of resting blood PCO_2.

Evidence supporting the role of glial cells in the mechanisms of central respiratory CO_2 chemoreception

The data showing that neuronal lesions or inhibition of the neuronal activity within a specific region of the brainstem leads to a reduction of the respiratory response to CO_2 does not necessarily mean that the sensitivity to H^+/CO_2 is an exclusive feature of neurons within that region. These neurons may relay signals from other chemoreceptor sites (for example, the RTN is known to receive inputs from the peripheral chemoreceptors in the carotid bodies (Takakura et al., 2006)) and/or their responses to changes in CO_2/pH are secondary and dependent on the responses triggered by these stimuli in neighbouring cells. There is significant evidence that the brainstem astrocytes and marginal glia are sensitive to changes in pH and CO_2 and play a significant role in the development of the respiratory responses to the chemosensory stimuli.

Studies in experimental animals (laboratory rats) showed that increases in the level of inspired CO_2 (systemic hypercapnia) trigger rapid release of ATP from the brainstem structures corresponding to the classical chemosensory areas of the ventral medullary surface (Gourine et al., 2005; Huckstepp et al., 2010b; Huckstepp et al., 2016). Blockade of ATP receptors was shown to inhibit the CO_2-induced increases in the activity

of individual respiratory neurons *in vivo* (Thomas & Spyer, 2000), responses of RTN neurons to acidification *in vitro* (Gourine et al., 2010; Wenker et al., 2010; Wenker et al., 2012), and overall respiratory responses to CO_2 *in vivo* (Thomas *et al.*, 1999; Gourine *et al.*, 2005; Wenker *et al.*, 2012). These data suggested that ATP mediates (at least in part) the effects of chemosensory stimuli on the activity of the brainstem respiratory network and pointed to the potential role of astrocytes, considering that brain glial cells (as many other non-excitable cells) use ATP as their main signalling molecule.

Earlier investigators had noted a dense glial layer covering the ventral surface of the brainstem at the locations corresponding to the classical chemosensory areas (Loeschcke, 1982) and the sites of chemosensory ATP release (Gourine et al., 2005). Recent studies of brainstem astrocytes revealed that the ventral medullary surface is populated by GFAP-positive cells with morphological features that are very different from that of a "prototypical" astrocyte (Sheikhbahaei et al., 2018a; van de Wiel et al., 2020). These cells have overlapping spatial domains and one main branching process projecting from the surface area deep into the brain tissue, reaching the respiratory networks of neurons (Figure 1). In this article we refer to these glial cells as projecting or marginal glia and propose that these projections may have a signalling function, as discussed in detail below. An extra layer of thin GFAP-positive processes appears between the ventral surface pial membrane and the parenchyma at the more rostral RTN level (Figure 1). Here, cell bodies of laminar GFAP-positive cells are located close to the pia mater and have numerous long processes coursing parallel to the ventral surface in the medio-lateral plane, creating a dense network of glial matter (Sheikhbahaei et al., 2018a) (Figure 1). This dense overlap of GFAP-positive fibres is not observed in any other brainstem region and represents a feature unique to the juxta ventral surface region of the medulla oblongata at the level of the RTN. It was reported that light stimulation of these glial cells transduced to express channelrhodopsin excites RTN neurons *in vitro* and stimulates the respiratory activity in anaesthetised rats *in vivo*, even if the experimental animals are kept below the apnoeic threshold by facilitated mechanical ventilation to reduce the arterial PCO_2 (Gourine et al., 2010). Thus, the effects of light-activation of astrocytes and marginal glia on individual RTN neurons and the respiratory activity *in vivo* were mimicking the effect of CO_2 and were found to be mediated by the release and actions of ATP (Gourine et al., 2010).

Additional evidence in support of the role of astrocytes in controlling the activity of the respiratory network and mediating the effect of CO_2 on breathing was obtained in animal models of Rett syndrome. Rett syndrome is a prototypical neurological disorder caused by loss of function of the transcriptional regulator methyl-CpG-binding protein 2 (MeCP2) gene (Amir et al., 1999). This condition is associated with a severely disordered breathing pattern and reduced ventilatory CO_2 sensitivity. Mouse models of Rett syndrome demonstrated that MeCP2 deficiency is associated with reduced respiratory responses to CO_2 (especially in conditions of mild and moderate hypercapnia), and elevated CO_2 apnoeic threshold (Toward *et al.*, 2013; Bissonnette *et al.*, 2014). MeCP2 is expressed in astrocytes (Yasui *et al.*, 2013; Forbes-Lorman *et al.*, 2014) and loss of MeCP2 function causes astrocytic defects (Okabe et al., 2012). Remarkably, re-introduction of the MeCP2 gene selectively in astrocytes was reported to rescue the normal respiratory pattern in MeCP2 deficient mice (Lioy et al., 2011). Moreover, conditional depletion of MeCP2 specifically in astrocytes (but not in neurons) resulted in a dramatic reduction of the ventilatory sensitivity to CO_2 (Garg et al., 2015). These results pointed to the importance of astrocytes for the operation of the respiratory network and its sensitivity to CO_2, but did not suggest a specific mechanism. It might be argued that MeCP2 deficiency affects the core function of astrocytes, for example in providing neurons with ionic and metabolic support, therefore, compromising the function of the respiratory neurons and their responses to the metabolic challenges. However, there is evidence that tonic and stimulated release of lactate (which in accordance with 'lactate shuttle hypothesis' mediates metabolic communication between astrocytes and neurons (Pellerin & Magistretti, 1994)) in the cortex and the brainstem was unaffected in MeCP2 knockout mice, while sensitivity of MeCP2 deficient astrocytes to changes in pH/PCO_2 was markedly impaired (Turovsky et al., 2015). These data provided evidence that MeCP2 deficiency may specifically affect the ability of astrocytes to detect the chemosensory stimuli.

In the next sections we discuss the cellular, membrane and molecular mechanisms underlying the sensitivity of the brainstem glial cells to H^+ and CO_2.

Sensing H^+

Ritucci and colleagues (Ritucci et al., 2005) first reported that RTN astrocytes are sensitive to changes in pH/PCO_2. In the experiments conducted in brainstem slices from

neonatal rats, the authors showed that CO_2-induced acidification (decrease in pH from 7.45 to 7.15) led to 4 mV (from −75 to −71 mV) and 5 mV (from −78 to −73 mV) depolarization of astrocytes recorded in whole-cell mode or using perforated-patch, respectively. These data were later confirmed by Wenker et al (Wenker et al., 2010) who reported that the membrane potential of pH-responsive RTN astrocytes in HCO_3^--buffered medium is between −75 and −82 mV, and that these astrocytes respond to chemosensory stimulation with a 9 mV depolarization; albeit a much stronger stimulus (pH 6.8) was used in that study. Results similar to the data obtained in these earlier studies were reported recently by the same research group (Patterson et al., 2021). Collectively, these studies described the membrane responses of RTN astrocytes to CO_2-induced acidification, however, the functional significance of relatively small membrane depolarizations (evoked in response to rather strong chemosensory stimuli) for astroglial function and signalling remains unclear.

Experimental studies involving the recordings of arguably the most important readout of astroglial activity, - intracellular Ca^{2+} (Bazargani & Attwell, 2016; Semyanov *et al.*, 2020), - demonstrated exquisite sensitivity of brainstem astrocytes and projecting glial cells to small changes in pH (Gourine et al., 2010). In the experiments conducted *in vivo* (laboratory rats) and *in vitro* (organotypic brainstem slices and acute brainstem slices of adult rats) glial cells that populate the ventral regions of the medulla, including the RTN, were found to sense decreases in pH as small as 0.2 units, responding to small shifts in acidic direction with robust elevations of intracellular [Ca^{2+}], leading to the release of ATP, which amplified/propagated Ca^{2+} excitation between the cells (Gourine et al., 2010). Recordings of vesicular fusion events in individual cultured rat brainstem astrocytes using total internal reflection fluorescence microscopy showed that ~35% of these cells respond to acidification with increased rate of exocytosis of ATP-containing vesicular compartments (Kasymov et al., 2013). These fusion events required intracellular Ca^{2+} signaling and were independent of autocrine ATP actions. In contrast, the rate of vesicular fusion in cultured cortical astrocytes was found to be insensitive to changes in pH (Kasymov et al., 2013). These data demonstrated that astrocytes in different brain regions are functionally specialized and provided one of the very first pieces of experimental evidence in support of the idea of astrocyte heterogeneity, – the concept of acute current research interest (Chai et al., 2017).

An initial pharmacological survey that evaluated the potential role of several putative pH-sensitive targets (including certain ion channels, transient receptor potential channels, and other membrane proteins) failed to suggest a plausible mechanism responsible for acidification-induced Ca^{2+} responses in brainstem astrocytes (Gourine et al., 2010). To aid identification of the underlying mechanism, the differences in gene expression between pH-sensitive brainstem astrocytes and pH-insensitive cortical astrocytes were analysed (Turovsky et al., 2016). The expression of several notable astroglial genes, including *SLC4a4* gene encoding electrogenic Na^+/HCO_3^- cotransporter 1 (NBCe1), *KCNJ10* and *KCNJ16* genes, encoding Kir4.1 and Kir5.1 subunits of inwardly rectifying K^+ channels, were found to be consistently higher in the brainstem (*vs* cortex) across different experimental conditions/developmental stages (Turovsky et al., 2016). Higher levels of Kir4.1 and Kir5.1 expression in the brainstem astrocytes (*vs* cortical astrocytes) was also confirmed by a recent study (Patterson et al., 2021)

Turovsky and colleagues (Turovsky et al., 2016) reported that Ca^{2+} responses in brainstem astrocytes induced by decreases in pH are preceded by Na^+ entry, reduced by pharmacological inhibition of Na^+/HCO_3^- cotransport (NBC) or Na^+/Ca^{2+} exchange (NCX), and abolished in Na^+-free medium or by combined NBC/NCX blockade. In NBCe1 deficient astrocytes, acidification-induced Ca^{2+} responses were largely absent (Turovsky et al., 2016). These data suggested that in pH-sensitive brainstem astrocytes, acidification activates NBCe1, which brings Na^+ inside the cell. Raising $[Na^+]_i$ activates NCX to operate in a reverse mode, leading to Ca^{2+} entry and Ca^{2+}-dependent exocytosis of ATP-containing vesicular compartments (Kasymov et al., 2013).

Thus, NCX reversal appears to be responsible for acidification-induced Ca^{2+} responses in pH-sensitive brainstem astrocytes. The results of electrophysiological studies discussed above showed that the membrane potential of these astrocytes is very close to the calculated reversal potential of NCX (−80 mV) (Kirischuk et al., 2012) set by a relatively high (15–20 mM) $[Na^+]_i$ in astrocytes (Kirischuk *et al.*, 2012;Parpura & Verkhratsky, 2012). Indeed, in pH-sensitive brainstem astrocytes, the resting $[Na^+]_i$ was found to be ~12 mM, increasing to ~20 mM in response to acidification (Turovsky et al., 2016). Schematic depiction of the mechanism underlying chemosensory Ca^{2+} responses in brainstem astrocytes is illustrated by Figure 2.

The functional significance of increased expression of inwardly rectifying Kir4.1 and Kir5.1 channels in pH-sensitive brainstem astrocytes (Turovsky *et al.*, 2016; Patterson *et al.*, 2021) requires further experimental clarification. Hawkins and colleagues (Hawkins et al., 2014) reported that conditional deletion of Kir4.1 specifically in astrocytes reduces the ventilatory response to CO_2. In a mouse model of Rett syndrome (characterised by markedly reduced respiratory CO_2 chemosensitivity, as discussed above), MeCP2 deficiency was found to be associated with a reduction of astroglial Kir4.1 expression (Kahanovitch et al., 2018) and impaired sensitivity (as assessed by recordings of Ca^{2+} responses) of brainstem astrocytes to CO_2-induced acidification (Turovsky et al., 2015). More recently it was reported that acidification-induced changes in the membrane conductance recorded in RTN astrocytes are reduced in conditions of Kir5.1 deficiency (Patterson et al., 2021). Rats that do not express Kir5.1 show a reduction in the ventilatory response to CO_2 (Puissant et al., 2019). However, in mice with global deletion of Kir5.1 central respiratory CO_2 chemosensitivity was reported to be normal (Trapp et al., 2011). It should be noted that interpretation of the data obtained in these studies is complicated by persistent metabolic acidosis observed in conditions of Kir5.1 deficiency in both animal models.

Sheikhbahaei et al (Sheikhbahaei et al., 2018b) studied the role of Ca^{2+}-dependent vesicular release of signalling molecules by astrocytes residing within the respiratory rhythm-generating circuits of the preBötC. Experiments were conducted in laboratory rats in which the preBötC astrocytes were transduced to express either the dominant-negative SNARE (dnSNARE) protein, light chain of tetanus toxin (TeLC), or a potent ATP-degrading enzyme transmembrane prostatic acid phosphatase (TMPAP) (Sheikhbahaei et al., 2018b). Both dnSNARE and TeLC were found to be equally potent in blocking Ca^{2+}-dependent vesicular release in brainstem astrocytes (Angelova et al., 2015; Sheikhbahaei et al., 2018b), whilst TMPAP expression was shown to effectively prevent ATP accumulation in astroglial vesicular compartments and block extracellular ATP actions (Marina et al., 2013; Wells et al., 2015). Sheikhbahaei et al (Sheikhbahaei et al., 2018b) reported that in conscious animals blockade of the vesicular release mechanisms in the preBötC astrocytes (expression of dnSNARE or TeLC) or facilitated degradation of extracellular ATP in the area (TMPAP expression) reduced the resting breathing rate and frequency of periodic sighs. Furthermore, dnSNARE or TeLC expression in preBötC astrocytes decreased the respiratory responses to CO_2 (by ~20%), and dramatically reduced the exercise capacity. These data suggested that

vesicular release of gliotransmitters by preBötC astrocytes provides the tonic excitatory drive to the respiratory rhythm-generating circuits. We propose that this tonic excitatory drive is the *CO_2 drive to breathe* exerted locally and mediated by astrocytes adjacent to the respiratory-rhythm generating circuits of the brainstem. The data reported by Sheikhbahaei et al (Sheikhbahaei et al., 2018b) also suggested that the key signalling molecule released by astrocytes to modulate the respiratory circuit activity is ATP, but there is also evidence that astroglial release of D-serine may contribute to the control of breathing in hypercapnic conditions (Beltran-Castillo et al., 2017).

Sensing CO_2

As an early study had demonstrated that ATP release from the ventral brainstem regions is an important initial event in the detection of chemosensory stimuli (Gourine et al., 2005), Huckstepp and colleagues tested the hypothesis that ATP could also be released via certain membrane channels (Huckstepp et al., 2010b). This is a plausible mechanism because ATP can permeate the pores of many different connexin hemichannels (Kang et al., 2008; Pearson et al., 2005; Stout et al., 2002). It was found that amongst different connexins, connexin 26 (Cx26) is preferentially expressed in the ventral medullary region, and in particular by the glial cells that populate the marginal zone (Huckstepp et al., 2010b). This evidence is consistent with several earlier studies suggesting that Cx26 is largely absent from neurons and is only expressed in a subset of glia, mainly those cells that are close to the margins of the brain parenchyma (Nagy *et al.*, 2011; Nagy & Rash, 2003; Nagy *et al.*, 2001; Rash *et al.*, 2001). This localisation of Cx26 hemichannels makes them a favoured candidate to release ATP. Studies in brainstem slices showing that a range of pharmacological agents with selectivity towards connexins blocked CO_2-dependent ATP release further supported this hypothesis (Huckstepp et al., 2010b). The same pharmacological agents, when used *in vivo* (application on the ventral surface of the brainstem in anaesthetised rats), reduced the respiratory responses to CO_2 by a similar degree as observed following the blockade of ATP receptors (~25%) (Gourine et al., 2005;Huckstepp et al., 2010b), and concomitantly reduced the CO_2-sensitive ATP release (Huckstepp et al., 2010b), supporting the involvement of connexin hemichannels in chemosensory control of breathing.

Huckstepp et al (Huckstepp et al., 2010a) reported that expression of Cx26 in HeLa cells was sufficient to endow them with a CO_2-sensitive conductance, which was not present in parental HeLa cells. Expression of Cx26 also permitted CO_2-dependent dye loading into, and CO_2-dependent ATP release from, HeLa cells (Huckstepp et al., 2010a). These experiments were performed in isohydric conditions (changes in PCO_2 were applied at constant extracellular pH), therefore, the actions of CO_2 were less likely to arise from intracellular acidification as this is well known to decrease the open probability of connexin hemichannels (Yu et al., 2007). In isolated membrane patches, in either the inside-out or outside-out configurations, allowing experimental control of pH on both sides of the membrane, CO_2 was found to gate Cx26 channel (Huckstepp et al., 2010a). The simplest interpretation of these data is that Cx26 is itself directly sensitive to CO_2. According to this hypothesis a direct interaction between CO_2 and Cx26 expressed by glial cells causes the hemichannels to open and release ATP to mediate adaptive changes in breathing via ATP-sensitive receptors expressed by neurons of the respiratory network. However, while this evidence is suggestive, it is not definitive. Conclusive evidence of a direct action of CO_2 on Cx26 channels requires full understanding of the binding and gating mechanisms as well as demonstration that mutations of Cx26 can change the sensitivity of the channel to CO_2.

Meigh et al (Meigh et al., 2013) used molecular phylogenetic analysis of Cx26 and related connexins to identify the motif that is important for CO_2 binding. Their starting point was that the three closely related members of the beta-connexin family: Cx26, connexin 30 (Cx30) and connexin 32 (Cx32), were previously shown to be CO_2-sensitive (Huckstepp et al., 2010a). In contrast, another member of beta-connexin family, – connexin 31 (Cx31), was found to be insensitive to CO_2 (Meigh et al., 2013). Given that these four connexins are closely related, but only three are CO_2-sensitive, differences in their amino acid sequences were explored to identify the structural features that could underlie the interactions of the channel with CO_2. The hypothesis that guided the sequence comparison was that CO_2 could potentially carbamylate a lysine residue (Huckstepp et al., 2010a). Carbamylation (also known as carboxylation) occurs spontaneously and is the formation of a labile covalent bond between the carbon atom of CO_2 and the nitrogen atom of the primary amine of the lysine side chain. This is a post-translational protein modification, the functional significance of which has largely been overlooked in mammalian physiology. CO_2 carbamylation was originally described as the basis of the Bohr effect, whereby CO_2 reduces the affinity of haemoglobin for O_2

(Kilmartin & Rossi-Bernardi, 1971). Carbamylation of lysine residues occurs in RuBisco (Lundqvist & Schneider, 1991), a key enzyme for photosynthetic carbon fixation, and also in microbial beta-lactamases (Golemi et al., 2001; Maveyraud et al., 2000). Given the ubiquity of CO_2 and the significance of CO_2 effects in living systems, George Lorimer (Lorimer, 1983) first proposed the hypothesis that carbamylation might be a general post-translational protein modification of high functional importance.

A comparison of the sequences of Cx26, Cx30, Cx31 and Cx32 identified K125 as being uniquely present in the CO_2-sensitive connexins (Meigh et al., 2013). K125 was present in a short motif (KVRIEG) that was absent from CO_2-insentive Cx31 (Meigh et al., 2013). An X-ray structure for Cx26 (Maeda et al., 2009), showed that this motif oriented K125 towards R104 of the neighbouring subunit of the hexamer. Indeed, in the X-ray structure these residues were found to be separated by a distance of only around 6Å (Maeda et al., 2009) (Figure 3). Carbamylation of K125 could potentially allow formation of a salt bridge between this residue and R104 to form a 'carbamate bridge' between subunits. Although these residues are not resolved in other structures of Cx26 (Khan *et al.*, 2020; Bennett *et al.*, 2016), the Cx26 structure predicted by Alphafold orients these residues somewhat differently from the X-ray structure, but nevertheless retains the proximity of K125 and R104 across the subunit boundary, which would allow formation of the inter-subunit carbamate bridge (https://alphafold.ebi.ac.uk/entry/P29033).

Strong experimental evidence points to the importance of K125 and R104 for the CO_2 sensitivity of Cx26. Transplantation of the carbamylation motif into Cx31, makes this connexin sensitive to CO_2 (Meigh et al., 2013). Results of the experiments involving targeted mutations of the residues K125 and R104 provide further support of this hypothesis. For example, K125R (arginine not being carbamylatable) abolished the CO_2 sensitivity of Cx26, as did R104A (removing the ability to make an inter-subunit salt bridge) (Meigh et al., 2013). The mutations K125E or R104E produced constitutively open channels that were no longer sensitive to CO_2 (Meigh et al., 2013). The mutation K125C resulted in a hemichannel that could be opened by NO via nitrosylation of the cysteine residue and formation of a bridge to R104 (Meigh et al., 2015), and the double mutation K125C and R104C resulted in a hemichannel that was found to be redox sensitive (Meigh et al., 2015). These studies also showed that hemichannels respond to changes in bath PCO_2 with "on" and "off" time constants of tens of seconds. However, this response time is likely to be a significant underestimate due to the time lag between

solution change (preparations were superfused in the bath in these experiments) and the actual change in CO_2 concentration in the vicinity of the K125 residues of the channel.

Collectively these data suggested that CO_2 has a direct action on Cx26, as mutations of the protein alter its sensitivity to CO_2 and/or confer channel sensitivity to other ligands. This mechanistic understanding allowed the development of a dominant negative protein, dnCx26, which carries two mutations, R104A and K125R. Studies utilizing fluorescence resonance energy transfer showed that dnCx26 assembles efficiently into hexamers with wild-type Cx26 (van de Wiel et al., 2020). HeLa cells that stably express Cx26 lose their CO_2 sensitivity when transfected to express dnCx26 (van de Wiel et al., 2020). dnCx26 is thus a very selective genetic tool to study the physiological role of Cx26 sensitivity to CO_2 in chemosensory control of breathing and other physiological processes.

van de Wiel and colleagues (van de Wiel et al., 2020) used lentiviral vectors to express either the wild type Cx26 or dnCx26 under the control of the GFAP promoter in the ventral regions of the medulla oblongata of adult mice. Expression of dnCx26 in an area called the caudal parapyramidal area (cPPy) reduced the CO_2 sensitivity of breathing. In animals transduced to express dnCx26 in this region, CO_2-induced increases in tidal volume and minute ventilation were reduced by ~30% compared to the responses recorded in animals transduced to express the wild type Cx26 (van de Wiel et al., 2020). The location of dnCx26 expression was found to be critical; when dnCx26 was expressed in ventral medullary regions either rostral to the cPPy (at the RTN level) or caudal to the cPPy, it had no effect on the ventilatory response to CO_2 (van de Wiel et al., 2020). Thus, Cx26-expressing CO_2-sensitive glial cells appear to be confined to a specific cPPy area of the medulla where they contribute to central respiratory CO_2 chemoreception.

Although no mutations that directly affect the carbamylation motif of Cx26 have been described in humans, there are several very rare dominant mutations that cause a severe pathological condition termed Keratitis-Ichthyosis-Deafness syndrome (KIDS) (Xu & Nicholson, 2013; Wilson *et al.*, 1991). There are 9 documented KIDS mutations and four of these A88V, N14K, N14Y and A40V abolish CO_2 sensitivity of Cx26 in a dominant fashion (Cook *et al.*, 2019; de Wolf *et al.*, 2017; de Wolf *et al.*, 2016; Meigh *et al.*, 2014). Although altered breathing control had not been described in KIDS

patients, two patients carrying the A88V mutation have been reported as experiencing central apnoea (Lilly et al., 2019; Meigh et al., 2014). These rare mutations support the idea that the CO_2-sensitive Cx26-mediated mechanisms contribute to the chemosensory control of breathing in humans.

K125 resides in the cytoplasmic loop of Cx26, therefore, CO_2 must cross the plasma membrane to reach this site. Historically, CO_2 was thought to diffuse through the plasma membrane freely. More recent evidence led to the understanding that the diffusion of CO_2 across the biological membranes is restricted and to large extent is mediated by certain membrane channels, such as aquaporin (AQP) water channels (Michenkova et al., 2021). While aquaporins are highly expressed in glial cells (e.g. AQP4), an intriguing possibility is that CO_2-sensitive connexins, including Cx26 and other beta-connexins, may function as both the sensors as well as conduits of effective CO_2 transfer across the membrane.

Chemosensory control of breathing by glial and neuronal mechanisms

What are the relative contributions of glial and intrinsic neuronal sensitivities to changes in pH/CO_2 in the brainstem mechanisms of chemosensory control of breathing? As discussed above, research of central respiratory CO_2 chemoreception is currently focusing primarily on understanding the functional role and mechanisms underlying pH sensitivity of RTN neurons (Guyenet et al., 2019). However, significant experimental evidence strongly supports the concept of a "distributed central chemosensitivity", which proposes that central respiratory sensitivity to CO_2 is mediated by multiple brainstem chemoreceptor sites (including the RTN, preBötC, raphe, cPPy and the others), with each site providing tonic excitation of the respiratory network in eucapnia and a fraction of the total response to hypercapnia (Nattie, 2000; Nattie & Li, 2012).

In support of the distributed central chemosensitivity hypothesis, the study by Sheikhbahaei et al (Sheikhbahaei et al., 2018b) provided evidence suggesting that at the level of the preBötC astrocytes mediate the CO_2 chemosensory drive to breathe. Results of earlier experimental studies in rats involving focal acidification of different brainstem regions indicated that contribution of the preBötC mechanism(s) to the overall respiratory response to CO_2 is ~20–25% (Nattie, 2000). This estimate matches the relative contribution of the preBötC astrocytes, suggested by the data obtained by

Sheikhbahaei et al (Sheikhbahaei et al., 2018b), who reported 20% and 23% reductions of the ventilatory response to CO_2 in conditions, when vesicular release mechanisms in preBötC astrocytes were blocked by TeLC or dnSNARE expression, respectively. From these data it is logical to conclude that the sensitivity of the respiratory rhythm-generating circuits of the preBötC to CO_2 is mediated by astrocytes (with the remaining ~80% of the total response mediated by other central chemoreceptor sites and peripheral mechanisms). Further support of this hypothesis is provided by several lines of experimental evidence indicating that ATP (released by astrocytes in a CO_2-sensitive manner) is a potent excitatory modulator of the preBötC network activity (Lorier *et al.*, 2007; Lorier *et al.*, 2008; Huxtable *et al.*, 2009; Huxtable *et al.*, 2010; Funk, 2013).

Chemosensory structures of the ventral brainstem surface regions located caudally from the RTN also mediate the effects of CO_2 on breathing. As discussed above, there is evidence that detection of CO_2 in the cPPy region is mediated by Cx26-expressing glial cells of the marginal layer that contribute ~30% of the ventilatory response to moderate hypercapnia (van de Wiel et al., 2020). The van de Wiel et al. study was conducted in animals with intact peripheral chemoreceptors, suggesting that the contribution of this mechanism to central respiratory CO_2 chemoreception is likely to be even more significant. The cPPy areas correspond to the more caudal chemosensory regions of the ventral surface of the medulla oblongata described by Loeschcke and colleagues (Loeschcke, 1982), and contain chemosensitive neurons (Ribas-Salgueiro et al., 2003;Ribas-Salgueiro et al., 2005). It is plausible that the CO_2 detection mediated by Cx26-expressing marginal glia contributes to, and/or converges with, pH detection mediated by the chemosensory neurons of the cPPy. The key glial cells in the cPPy are GFAP-expressing projecting glial cells with large cell bodies located close to the brainstem surface and long branching processes that project both rostrally and medially (van de Wiel et al., 2020) (Figure 1f). These long processes may be able to signal, presumably via propagating Ca^{2+} excitation and release of gliotransmitters, to dorsally located neurons within the respiratory network. In this regard, neurons of the ventral respiratory column and raphé regions are within reach of these cells, giving the possibility that they may modulate the activities of these neurons in a CO_2-sensitive manner.

But what is the contribution of pH-sensitive glia and ATP release to central respiratory CO_2 chemoreception at the level of the RTN? In anaesthetised and conscious rats with

intact peripheral chemoreceptors, blockade of ATP receptors in the RTN following the application of P2 receptor antagonists on the ventral surface of the medulla, or after bilateral microinjections directly into the RTN, led to a reduction of the ventilatory response to CO_2 by 30-40% (Gourine *et al.*, 2005; Wenker *et al.*, 2012; Barna *et al.*, 2016). Perhaps in order to answer the above question it is prudent to compare the effects of ATP receptor blockade within the RTN and the effects of direct and specific inhibition of the RTN neurons. It was reported that genetic targeting and silencing of RTN neurons bilaterally inhibits the ventilatory sensitivity to CO_2 by ~30% and 60% in anaesthetised and conscious rats, respectively (Marina et al., 2010). Significantly larger effects were reported by Souza and colleagues (Souza et al., 2018) who observed ~90% reduction of the ventilatory response to CO_2 following near complete lesions of RTN neurons after bilateral microinjections of neurotoxin substance P-saporin conjugate in rats. Thus, silencing or lesions of RTN neurons appear to have a larger effect on the ventilatory response to CO_2, when compared to the effects of pharmacological ATP receptor blockade in the region. However, it is important to note that saporin is a potent astrocyte toxin which produces glial damage within and at some distance from the immediate site of the injection (Lin et al., 2013). Therefore, glial damage may have potentially contributed to the respiratory phenotype observed by Souza et al following substance P-saporin microinjections into the RTN (Souza et al., 2018). Furthermore, lesions or inhibition of the neuronal activity in the RTN would be expected to interrupt the relay of chemosensory information from the periphery (Takakura et al., 2006) and/or other central sites (Wu et al., 2019). Therefore, the effects of the experimental manipulations of this type are likely to overestimate the relative contribution of the intrinsic chemosensitivity of the RTN neurons to the development of the ventilatory response to CO_2.

Studies of the potential mechanisms underlying intrinsic chemosensitivity of the RTN neurons suggested that responses of these neurons to changes in pH are mediated by the proton-activated receptor GPR4 and the pH-sensitive K^+ channel TASK-2 (Kumar et al., 2015). Acidification-induced responses in a subset of RTN neurons and ventilatory responses to CO_2 in conscious mice were reported to be markedly reduced in conditions of global GPR4 deficiency, and almost complete blockade of the CO_2-induced response was observed in conditions of global constitutive GPR4 and TASK-2 double knockout (Kumar et al., 2015). However, a separate study conducted in GPR4 reporter mice showed a rather limited expression of this receptor by the RTN neurons (no glial

expression of GPR4 was observed) (Hosford et al., 2018). Moreover, systemic and/or central blockade of GPR4 using a highly potent lipophilic GPR4 antagonist (NE 52-QQ57) had no effect on CO_2-induced respiratory responses in anaesthetised rats, and a relatively small effect (reduction of the CO_2 response by ~20% on average) of pharmacological GPR4 blockade was observed in conscious rats and mice (Hosford et al., 2018). Thus, further studies seem to be required in order to clarify the role of GPR4 receptors in the mechanisms of central respiratory chemosensitivity.

Another plausible mechanism of how glial release of ATP in the ventral regions of the medulla may contribute to the development of the ventilatory response to CO_2 was suggested by the data reported by Mulkey and colleagues (Hawkins *et al.*, 2017; Cleary *et al.*, 2020). Hawkins et al (Hawkins et al., 2017) showed that in contrast to cortical blood vessels, RTN vasculature constricts in response to CO_2 and this constriction is mediated by ATP. The authors proposed that vasoconstriction induced by ATP slows CO_2 washout from the region, promotes local extracellular acidification, and amplifies the RTN neuronal responses. A recent study by the same group showed that the vascular responses in the RTN are mediated by $P2Y_2$ receptors, and when $P2Y_2$ receptors are deleted from the vascular smooth muscle cells, the ventilatory response to CO_2 is reduced by ~30% (Cleary et al., 2020).

The relative contribution of pH and CO_2 sensitive glial mechanisms to chemoreception at different brainstem sites and at different levels of hypercapnia requires further clarification. The latest study by van de Wiel and colleagues (van de Wiel et al., 2020) reported that Cx26-mediated mechanisms play a more prominent role at modest levels of CO_2, suggesting that different mechanisms may contribute differently depending on the strength of the chemosensory stimulus. We hypothesise that the Cx26-mediated mechanism of direct CO_2 sensing is responsible for the initial ATP release and at moderate levels of hypercapnia. Vesicular release mechanisms in chemosensory glial cells are hypothesised to be recruited subsequently and at higher levels of CO_2, in particular in conditions when CO_2-induced intracellular acidification would be expected to reduce the open probability of the connexin channels. Functional interactions and some 'overlap' between these two mechanisms may also exist. Although NBCe1-mediated Na^+ entry and NCX reversal appear to be largely responsible for acidification-induced Ca^{2+} responses in pH-sensitive brainstem astrocytes (Turovsky et al., 2016), it

is also possible that in cells expressing Cx26, Ca^{2+} influx through Cx26 channel could trigger the vesicular release.

Our current understanding of the relative significance and the mechanisms of glial and neuronal responses to changes in pH and CO_2 in the brainstem regions responsible for the chemosensory control of breathing allows us to propose the following unifying hypothesis. In this system the controlled variables are the brainstem tissue pH and PCO_2, which are determined by the metabolic production of protons and CO_2 by the brainstem cells, chemical composition (pH, PCO_2, $[HCO_3^-]$) of the arterial blood entering the brain, and the rate of blood flow through the brainstem. The available evidence suggests that the basic pH sensitivity of the respiratory network is mediated by the intrinsic neuronal mechanisms that in response to acidification increase the firing rate of neurons that constitute (preBötC) and/or project (RTN, cPPy, raphe) to the respiratory circuits. The chemosensory responses induced concomitantly in glial cells adjacent to and intermingled with these neuronal populations are essential for the full expression of the ventilatory response to hypercapnia. Astrocytes and the projecting marginal glial cells contribute to central respiratory CO_2 chemoreception via three distinct mechanisms: 1) acidification and CO_2-induced vesicular and Cx26-mediated release of ATP (and potentially other signalling molecules) which provide excitatory modulation of the respiratory network, 2) increased uptake of bicarbonate, resulting in a stronger extracellular acidification and, therefore, enhanced chemosensory neuronal response, which may be particularly significant at the RTN level, and 3) acidification and CO_2-induced vesicular and Cx26-mediated release of ATP which constricts brainstem blood vessels, slows CO_2 washout, maintains extracellular acidification, and, thereby, also enhances the RTN neuronal response. We propose that all these mechanisms working in concert are responsible for exquisite sensitivity of the respiratory network to CO_2. This hypothesis represents further development of the ideas communicated by the authors of this essay in earlier review articles (Gourine, 2005; Huckstepp and Dale, 2011; Marina et al., 2018) and increasingly recognized by other investigators in the field (Guyenet et al., 2019).

Role of astrocytes in regulation of local brain tissue pH

Brainstem astrocytes and marginal glia that are projecting and adjacent to, and/or intermingled with, the neuronal respiratory control circuits can modulate breathing

directly and, therefore, contribute to the maintenance of systemic (arterial) pH homeostasis. However, the chemosensitive neurons and glia of the brainstem cannot possibly detect and control changes in tissue pH and CO_2 in other parts of the brain, which may occur locally, for example in response to changes in the neuronal circuit activity.

A recent study by Theparambil et al (Theparambil et al., 2020) suggested the existence of another key astroglial mechanism, essential for the maintenance of local brain tissue pH in face of variable extracellular acid loads that depend on neuronal activity. Recordings of intracellular pH in cortical astrocytes *in vivo* (anaesthetised mice) and *in vitro* (acute brain slices) showed that in response to the increases in the neuronal activity ~30% of all astrocytes release bicarbonate to buffer extracellular H^+ loads associated with neuronal activation (Theparambil et al., 2020). Interestingly, release of ATP and ATP-mediated signalling appear to play a central role in the operation of this mechanism. There is significant evidence that increased neuronal activity is associated with the release of purines (Dale *et al.*, 2002; Pascual *et al.*, 2005; Pankratov *et al.*, 2006; Gourine *et al.*, 2008; Wall & Dale, 2013; Sims & Dale, 2014; Wells *et al.*, 2015; Badimon *et al.*, 2020; Peng *et al.*, 2020). Theparambil et al (Theparambil et al., 2020) reported that ATP and ADP (ATP is rapidly broken down to ADP by ectonucleotidase activity in the extracellular space) trigger bicarbonate secretion by cortical astrocytes via activation of $P2Y_1$ receptors, recruitment of phospholipase C, release of Ca^{2+} from the internal stores, and facilitated outward HCO_3^- transport by the sodium bicarbonate transporter NBCe1. Astrocytes characteristically express high levels of NBCe1 (Zhang *et al.*, 2014; Theparambil *et al.*, 2020), which is a high affinity carrier primarily responsible for transporting HCO_3^- across the astroglial membrane (Theparambil et al., 2014). In mice with astrocyte-specific conditional knockdown of NBCe1, extracellular pH in the cortex is not well maintained, and neuronal activation results in a significant extracellular acidification (Theparambil et al., 2020). In contrast to the brainstem glia, the properties of cortical astrocytes and NBCe1 expressed by these cells favour the outward activity of the transporter, as discussed in detail in (Theparambil et al., 2020).

Collectively these data suggest that astrocytes in the regions of the brain other than the brainstem are equipped with a mechanism that effectively counteracts extracellular acidification and maintains the extracellular pH homeostasis locally, via the NBCe1-mediated release of bicarbonate 'on demand' and in accord with the prevailing levels of

local neuronal activity and energy usage. Schematic depiction of the astroglial mechanism responsible for regulation of local brain tissue pH is illustrated by Figure 4.

Clinical studies have demonstrated that homozygous mutations of *Slc4a4* gene (associated with variable degree loss of NBCe1 function) result in permanent renal tubular acidosis, glaucoma and hemiplegic migraine (Horita et al., 2005; Suzuki et al., 2010). Heterozygous carriers of *Slc4a4* mutation may also display some of these pathological features (Suzuki et al., 2010). Mouse models showed that NBCe1 activity is critically important for homeostasis, as global NBCe1 knockout animals develop severe metabolic acidosis and do not survive beyond the third week of life (Gawenis et al., 2007). Breathing deficits and aberrant brain pH regulation may contribute to the development of this harmful phenotype, as discussed previously (Turovsky *et al.*, 2016; Theparambil *et al.*, 2020).

Role of astrocytes in mediating the effects of CO_2 on cerebral blood vessels

Dilation of cerebral blood vessels and increase in brain blood flow in response to CO_2 represent another important mechanism that contributes to the maintenance of brain pH homeostasis. In the brain, most CO_2 is generated by neurons (Howarth et al., 2012), therefore, CO_2/acid production by the brain tissue increases in parallel with neuronal activity and energy usage. High vascular reactivity to CO_2 is a unique feature of cerebral blood vessels (Ainslie et al., 2005; Willie et al., 2014), with grey matter microvasculature displaying the greatest CO_2 sensitivity (Noth et al., 2008; Willie et al., 2014). Considering the operation of highly effective cardiorespiratory mechanisms which maintain constant levels of arterial and brainstem pH and PCO_2 (Ainslie & Duffin, 2009; Spyer & Gourine, 2009), the evolutionary development of the mechanism(s) responsible for high CO_2 sensitivity of all contractile cerebral vessels points to its high adaptive value in ensuring effective CO_2 removal, essential for the maintenance of constant brain tissue pH.

There are hundreds of studies suggesting the existence of multiple signaling pathways that can potentially mediate the effects of CO_2 on brain blood flow. The largest number of published studies targeted (pharmacologically or genetically) the signalling pathways mediated by nitric oxide and cyclooxygenase products. Recruitment of these pathways appear to mediate the effects of CO_2 on cerebral vasculature (Hoiland et al., 2019). It

is of interest to note that the same vasodilatory signaling pathways (mediated by NO and cyclooxygenase products) are also implicated in the responses of cerebral blood vessels to the increases in the neuronal activity (Hosford & Gourine, 2019), supporting the oldest, but largely discounted, hypothesis that metabolic communication between neurons and cerebral vasculature (underlying the development of the neurovascular coupling response) is mediated by CO_2 (Roy & Sherrington, 1890; Hosford et al., 2020). However, the cellular and molecular mechanisms of cerebrovascular CO_2 *sensing* remain largely unknown.

Howarth and colleagues (Howarth et al., 2017) reported data suggesting that astrocytes mediate the dilations of cerebral blood vessels in response to CO_2. The authors showed that cortical astrocytes respond to CO_2 with elevations in intracellular Ca^{2+}, activation of cyclooxygenase-1, increased production and release of prostaglandin E_2, leading to the dilation of the blood vessels associated with these astrocytes (Howarth et al., 2017). The same study reported that generation of prostaglandin E_2 by astrocytes and cerebrovascular responses to CO_2 depend on the level of glutathione in the brain (Howarth et al., 2017). However, the data reported by Howarth et al (2017) are not supported by the results of earlier studies showing that in contrast to the brainstem astrocytes, cortical astrocytes do not appear to display clear responses to chemosensory stimuli (Gourine et al., 2010; Kasymov et al., 2013; Turovsky et al., 2016). Other studies reported that a prototypical glial transmitter ATP can trigger capillary dilations via relaxation of pericytes (Mishra et al., 2016) and propagating dilations of cerebrovasculature via signalling to the capillary endothelial cells (Thakore et al., 2021). However, CO_2-induced release of ATP in the brain regions other than the brainstem is yet to be documented. Thus, in the opinion of the authors further experimental studies are required to determine the role played by astrocytes in mediating the effects of CO_2 on cerebral blood vessels.

Conclusions

Throughout life, behaviour, metabolism, and brain function constantly vary with activity. Such variations have the capacity to have a major impact on brain acid-base balance. Yet, the brain pH homeostasis is very well maintained in face of variable extracellular acid loads that depend on changes in the neuronal activity and metabolism, and also in conditions of major acute or chronic (physiological or pathological)

perturbations of systemic acid-base balance (Mitchell et al., 1965). In this article we have discussed the cellular and molecular mechanisms underlying the sensitivity of astrocytes and marginal glial cells to changes in pH and CO_2 and reviewed the evidence suggesting that these cells are critically important for the operation of the multiplicity of mechanisms (local and systemic) that work in concert to keep the brain pH constant.

There is emerging evidence that brain aging is associated with progressive tissue acidification and this process is facilitated during the development of the neurodegenerative disease, such as Alzheimer's disease (Lyros *et al.*, 2020; Decker *et al.*, 2021). It is conceivable that impaired function of the mechanisms discussed in this essay may contribute to age-related brain tissue acidification with detrimental effects on synaptic function, neuronal activity, and neuronal viability. If this hypothesis is correct, then full understanding of the mechanisms of brain H^+/CO_2 sensing and control may prove to be important for the development of preventive and therapeutic strategies to maintain cognitive health and promote brain longevity.

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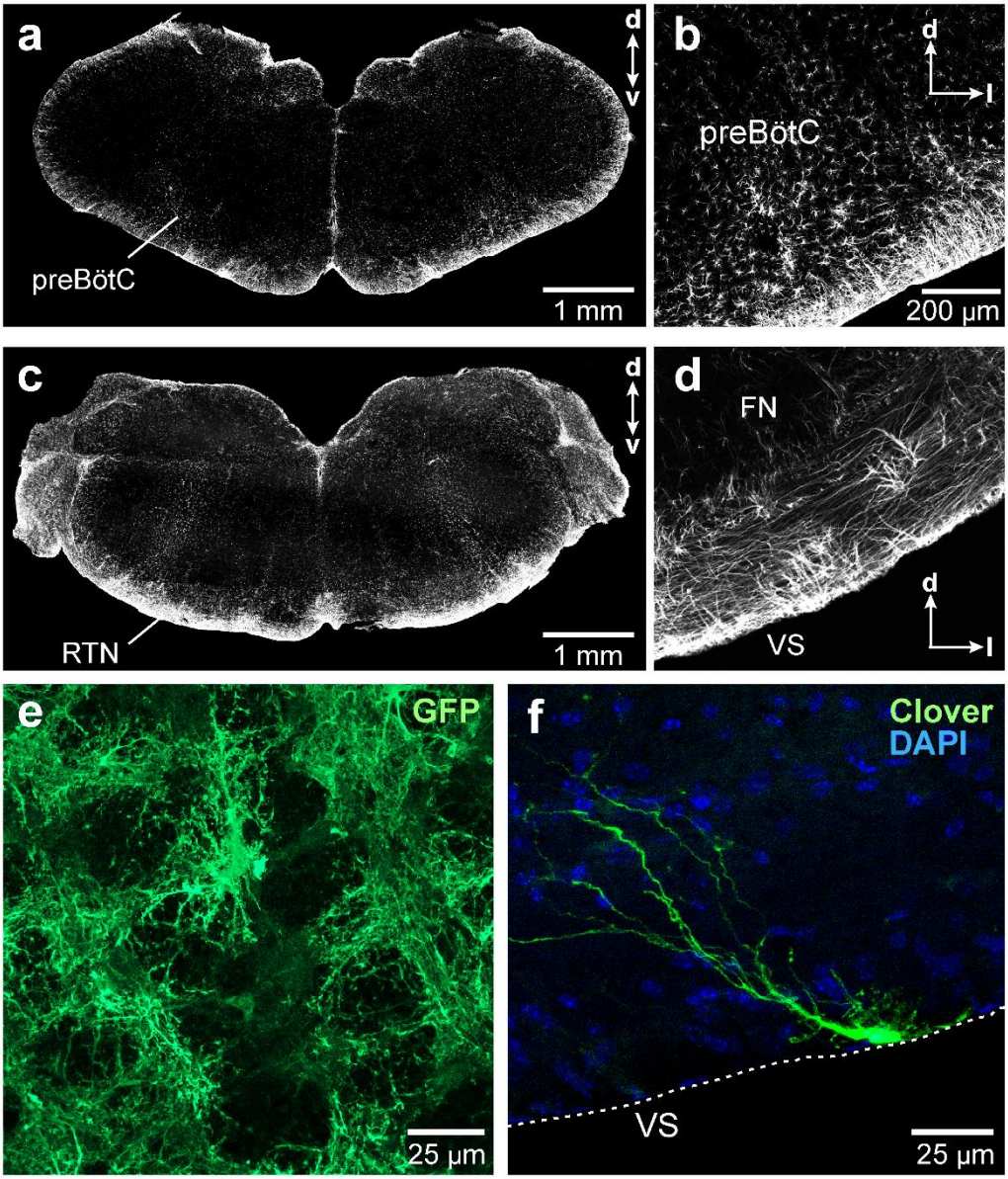

Figure 1| Glial cells of the ventral regions of the brainstem. (**a**) Tiled low magnification confocal image of the coronal section of the brainstem taken at the level of the pre-Bötzinger complex (preBötC) illustrating distribution of glial fibrillary acidic protein (GFAP)-positive cells; (**b**) Higher magnification image of the ventral aspect of the medulla oblongata illustrating GFAP-positive astrocytes and the marginal glia at the level of the preBötC; (**c**) Tiled low magnification confocal image of the coronal section of the brainstem taken at the level of the retrotrapezoid nucleus (RTN) illustrating the distribution of GFAP-positive cells; (**d**) High magnification image of the RTN laminar glial cells forming a dense network of GFAP-positive processes at the ventral brainstem surface (VS); (**e**) Confocal image illustrating astrocytes of the preBötC transduced to express green fluorescent protein (GFP) under the control of the GFAP promoter; (**f**) Confocal image illustrating a representative example of a projecting marginal glial cell transduced to express Clover under the control of the GFAP promoter. d, dorsal; l, lateral; v, ventral; FN, facial nucleus. We thank Drs Shahriar Sheikhbahaei and Joseph van de Wiel for providing the images. Panels **a**-**d** are adapted from (Sheikhbahaei et al., 2018a).

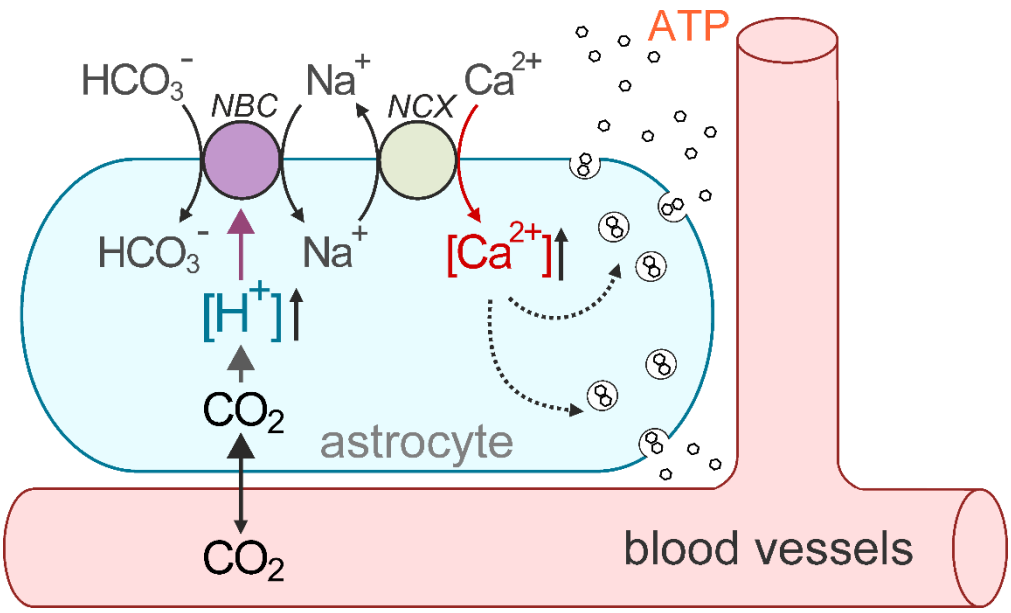

Figure 2| Schematic illustration of the plausible mechanism underlying chemosensory Ca^{2+} responses in brainstem astrocytes. In this specialized population of chemosensory glia, CO_2-induced intracellular acidification stimulates inward activity of the Na^+/HCO_3^- cotransporter (NBC), which brings Na^+ inside the cell. Increased $[Na^+]_i$ activates Na^+/Ca^{2+} exchanger (NCX) to operate in a reverse mode, leading to Ca^{2+} entry, and facilitated vesicular exocytosis of ATP. Illustration adapted from (Turovsky et al., 2016).

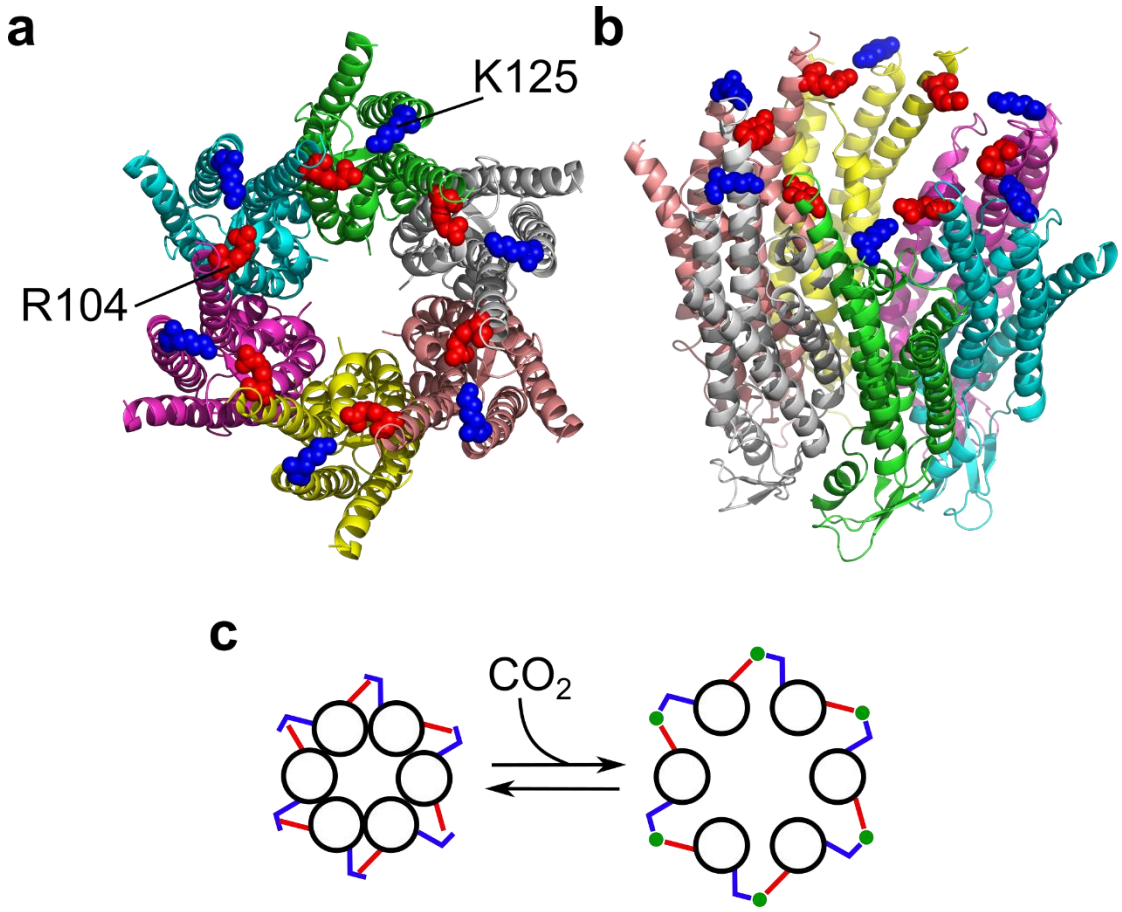

Figure 3| X-ray crystal structure of Cx26 (2zw3 from the protein database) showing the position of residues K125 (blue) and R104 (red) that are proposed to form the carbamate bridge. (**a**) View of the channel pore from the cytoplasmic face along the axis of the pore; (**b**) Side view of the channel showing that R104 and K125 are in a similar plane. The 2zw3 structure (obtained in the absence of CO_2) shows the channel in the open configuration (Maeda et al., 2009). R104 and K125 of adjacent subunits orient towards each other and are 6.5 Å apart. Carbamylation of K125 by CO_2 forms a carbamate bridge between subunits that leads to the opening of the channel; (**c**) Cartoon representation of CO_2 binding (green circles) to K125 (blue) and formation of the carbamate bridge to R104 (red) leading to channel opening. While the exact nature of the conformational change leading to channel opening is unclear, it is hypothesised to involve movement of the N termini of the 6 subunits which individually are in a helical secondary conformation and interact to form a plug within the pore (Maeda *et al.*, 2009; Brotherton *et al.*, 2020).

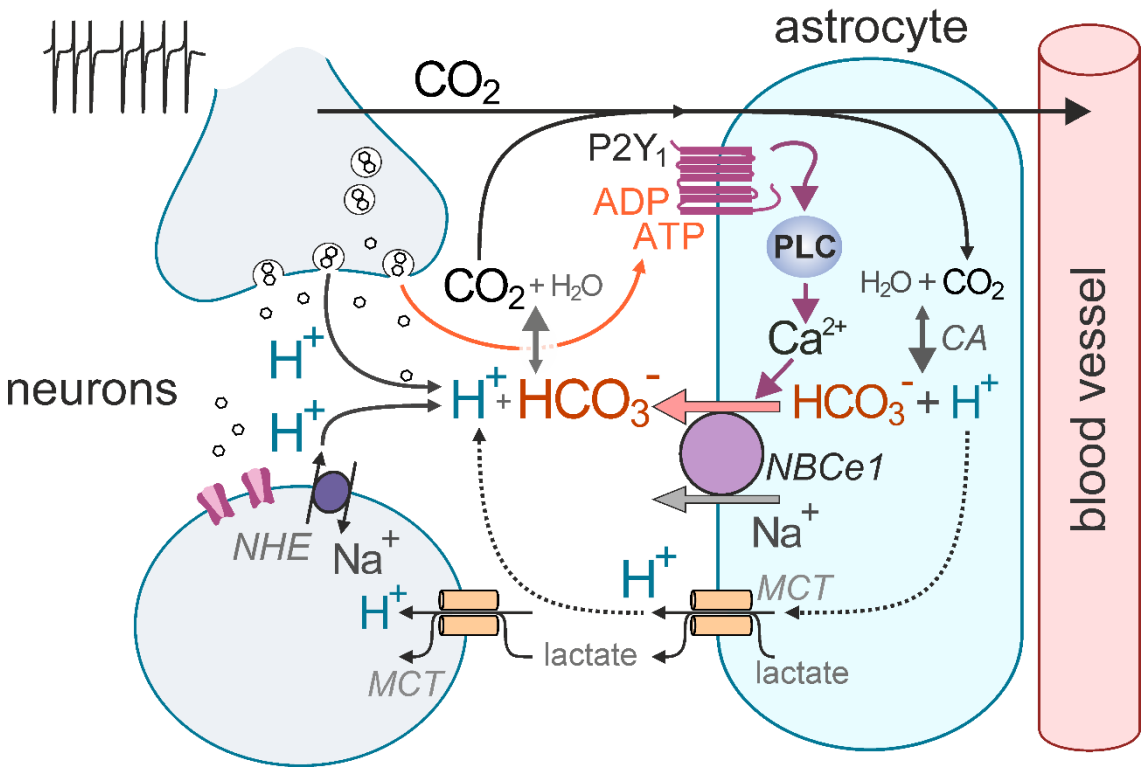

Figure 4| Schematic drawing of the neurovascular unit illustrating the hypothesised astroglial mechanism contributing to the maintenance of brain extracellular pH locally. Neuronal activation is associated with the release of ATP which is rapidly broken down to ADP by ectonucleotidase activity in the extracellular space. In cortical astrocytes, ATP/ADP trigger bicarbonate secretion via activation of $P2Y_1$ receptors, recruitment of phospholipase C (PLC), release of Ca^{2+} from the internal stores, and facilitated outward HCO_3^- transport by the sodium bicarbonate cotransporter NBCe1. It is proposed that extracellular acid loads associated with enhanced neuronal activity are buffered by HCO_3^- released by astrocytes via this mechanism. Note that in contrast to brainstem astrocytes, in the majority of cortical astrocytes NBCe1 operates in the outward mode. Also illustrated are the sources of extracellular acid loads, including (but not limited to) monocarboxylate transporter (MCT)-mediated co-transport of H^+ and lactate, exocytosis of acidic neurotransmitter vesicles, and operation of H^+ extruders, such as Na^+/H^+ exchanger (NHE). Illustration adapted from (Theparambil et al., 2020).